# Heterologous Expression of Terpene Synthase Enzymes in *Methanosarcina*

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## Abstract

Methanogens are obligate anaerobes that utilize inexpensive, non-food substrates and make methane as a by-product. Previously in this lab, methanogens have been engineered to direct carbon to isoprene production. Methanogens then could be engineered to make terpenes which are made of several modified isoprene units. Terpenes are molecules of interest to engineer because they are currently synthesized from non-renewable petroleum or are harvested from their endogenous species at low expression levels. Terpenes are part of multi-billion dollar industries like flavoring, fragrance, pharmaceuticals, and have potential in the energy industry.

Methanogens are being investigated for engineering terpenes because they don't exihibit feedback inhibition in their mevalonate (MVA) pathway where terpene precursors come from. A higher percentage of their carbon could be directed to the product of interest than in other model species used for engineering. This project aims to reduce fossil fuel use and increase renewable energy by engineering methanogens to sustainably synthesize terpene compounds.

## Research Questions

- Can *Methanosarcina acetivorans* synthesize farnesyl diphosphate, geraniol, linalool, sabinene, pinene, bisabolene, valencene, and humulene from inexpensive non-food feedstocks such as $CO_2$, methanol, or acetate?
- Can these terpenes be synthesized in appreciable amounts?
- Are these terpenes toxic to *M. acetivorans*?

## Terpene Synthases

- Methanogens have a naturally high-flux MVA pathway for isoprenoid lipids compared to model organisms.
- Terpenes are part of billion dollar industries of fragrance and flavoring.
- Terpenes are currently derived from native organisms or petroleum.
- Terepenes have potential in the pharmaceutical, materials, and energy industries:
  - Fuel blending.
  - Sustainable jet fuel with thermal stability and low pressure tolerance.

**Figure 1:** Chemical structure of terpene compounds of interest for production in *M. acetivorans*.

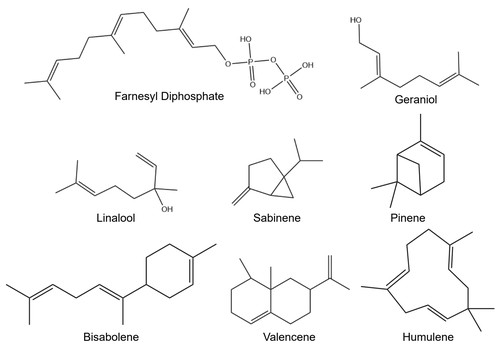


**Table 1:** Terpene enzyme names, plasmid names, and *M. acetivorans* strain numbers containing the corresponding terpene gene.

| Enzyme | Plasmid Name | Strain Number |
|---|---|---|
| (2E,6E)-farnesyl diphosphate synthase | pSC12 | NB652, NB692 |
| Geraniol synthase | pSC13 | NB693, NB695 |
| S-linalool synthase | pSC14 | NB697, NB698 |
| (+)-sabinene synthase | pSC17 | NB653, NB654 |
| Pinene synthase | pSC18 | NB700, NB701 |
| Farnesyl diphosphate synthase | pSC19 | NB703, NB704 |
| Alpha-bisbolene synthase | pSC23 | NB705, NB706 |
| Valencene synthase | pSC35 | NB696 |
| Alpha-humulene synthase | pLK8 | NB707, NB708 |

## Cloning and Transforming Terpene Expression Plasmids

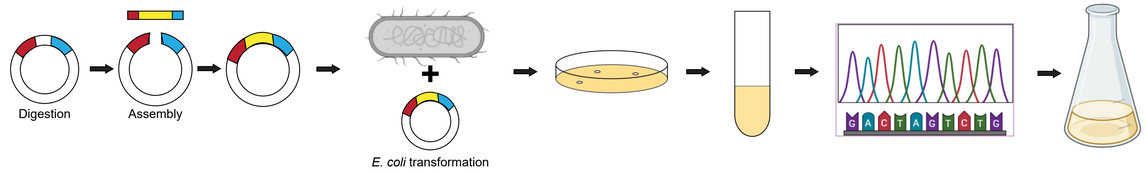


**Figure 2:** Plasmid assembly using NEB HiFi on digested backbone and terpene gene. Transform plasmid into *E. coli* via heatshock and plate. Select colonies into LB media with ampicillin. Harvest plasmids, restriction digest screen, and sequence. Correct plasmids are re-transformed and harvested in bulk for methanogen transformations.

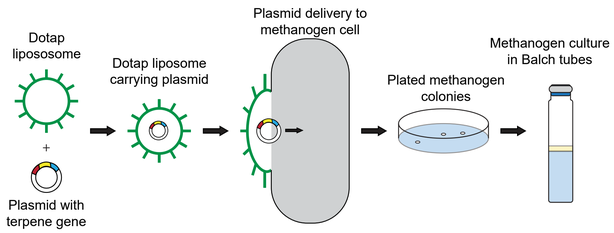


**Figure 3:** Method of transforming methanogens with terepene plasmids. Successful colonies are streaked for isolation then grown in Balch tubes containing high-salt media with a layer paraffin oil layer.

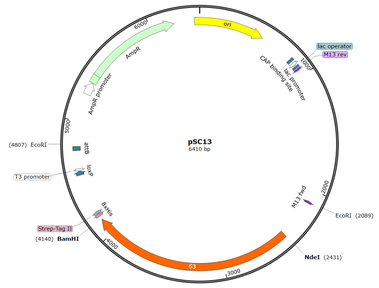


**Figure 4:** Map of pSC13. Backbone is pNB730 digested with NdeI and BamHI, and the insert is a terpene gene optimized for *M. acetivorans*. His tag is expressed at end of the terpene gene.

## Strain Validation

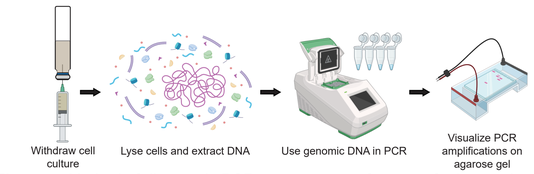


**Figure 5:** Method of diagnostic PCR to screen transformants for integration of terpene gene into the methanogen genome.

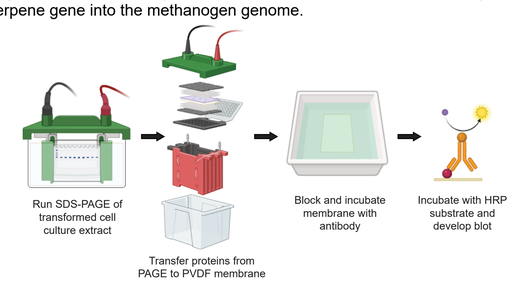


**Figure 6:** Method of Western blot to detect if the His-tagged terpene enzyme is being produced in the cell.

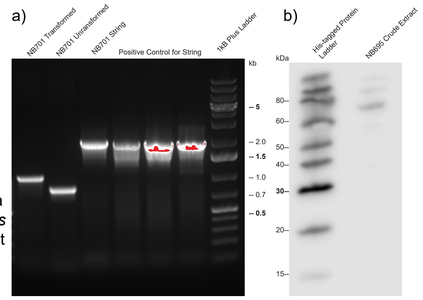


**Figure 7:**
a) Agarose gel PCR screen of pSC18 *M. acetivorans* (NB701) transformant. Lane 3 shows a band for the string at 2kb like the positive controls.
b) Western blot with a pSC13 *M. acetivorans* (NB695) crude extract band 60-80 KDa. Expected size is 65kDa.

## Toxicity Tests

**Figure 8:** Toxicity determined by adding pure terpene to a normal *M. acetivorans* culture and measuring optical density over time.

**Results:**
- 20mM geraniol has no significant effect on growth rate (p=0.64) or carrying capacity (p=0.64).
- 20mM valencene has no significant effect on growth rate (p=0.30) or carrying capacity (p=0.83).

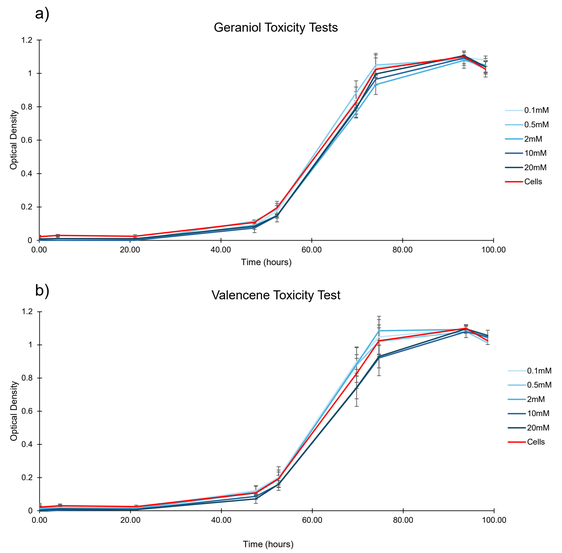


**Figure 9:** Growth curves of geraniol (a) and valencene (b) over a time period of 100 hours. Methanol media tubes are inoculated with untransformed *M. acetivorans* and paraffin oil containing the terpene. Incubated at 35°C and measured on Spectronic 20 at λ=600nm. N=3

## Next Steps

- Toxicity tests using farnesyl diphosphate, linalool, sabinene, pinene, bisabolene, and humelene.
- Continue Western blots for farnesyl diphosphate, linalool, sabinene, valencene, pinene, bisabolene, and humelene strains.
- RT-qPCR
- Enzyme Assays
- Gas Chromatography

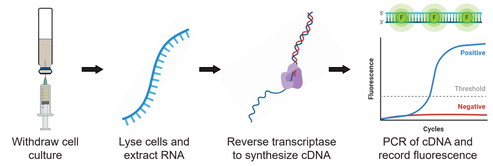


**Figure 10:** RT-qPCR procedure to check transcription of the terpene genes.

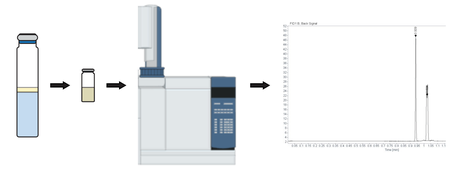

**Figure 11:** Gas chromatography is performed on the paraffin oil from stationary phase culture. Compare gas chromatogram to the standard terpene in paraffin oil to identify terpene quantities made by methanogens.

## Acknowledgements

This work was supported by the Nebraska Public Power District through the Nebraska Center for Energy Sciences Research at the University of Nebraska-Lincoln.

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